INHIBITOR OF OSTEOCLAST PRECURSOR FORMATION

This invention relates to a polypeptide factor which is able to inhibit the formation of osteoclasts. In particular, the invention relates to a factor which inhibits the differentiation of haematopoietic precursor cells into mononucleate osteoclast precursors. In a preferred form of the invention, the factor is a type II membrane polypeptide expressed on the osteoblast cell surface, which we have designated osteoclast inhibitory lectin (OCIL).

BACKGROUND OF THE INVENTION

In normal adults, the processes of bone formation and resorption are balanced in order to maintain a normal healthy bone mass. With the onset of the menopause in females and with ageing in both sexes, the rate of bone resorption exceeds that of bone formation, resulting in net bone loss, and ultimately in osteoporosis.

Osteoblasts are the bone cells responsible for bone formation, while osteoclasts are responsible for resorption of bone. Our understanding of the factors that regulate the formation and function of osteoclasts has been greatly enhanced by laboratory methods that have enabled us to isolate and grow these cells in culture. It is now well established that the development of active osteoclasts *in vitro* requires intimate contact between osteoblastic stromal cells and precursors of osteoclasts which are derived from haematopoietic cells belonging to the monocyte/macrophage lineage (Takahashi *et al*, 1988). This process is influenced by a variety of factors, including 1,25-dihydroxyvitamin D_3, parathyroid hormone, prostaglandin E_2, and interleukins 6, 11 and 17, all of which enhance osteoclast formation. In contrast, cytokines such as interleukins 4, 10, 13 and 18 are inhibitory (Suda *et al*, 1995; Martin and Udagawa, 1998).

All factors which stimulate osteoclast formation

act directly on the osteoblast population and not on the osteoclast precursors, leading to the proposal that osteoblasts or stromal cells express a membrane-associated peptide that regulates the formation of functional multinucleate osteoclasts. A factor, termed "Osteoclast Differentiation Factor" (ODF), that fulfils the functions of such a putative membrane-associated peptide has recently been cloned. ODF encodes a 316 amino acid type II transmembrane protein, and is a member of the TNF ligand family (Yasuda *et al*, 1998). Recombinant protein corresponding to the extracellular domain of ODF stimulates the formation of active, bone-resorbing osteoclasts from haematopoietic cells within the spleen, even in the absence of stromal cells. A peptide identical to ODF has also been cloned from T cells, and designated Tumour Necrosis Factor-related activation-induced cytokine (TRANCE; Wong *et al*, 1997), or receptor activator of NF-κB ligand (RANKL; Anderson *et al*, 1997). When released by T cells following activation of the T cell receptor, it mediates the interaction of T cells and dendritic cells, resulting in stimulation as well as increased survival of the naïve T cells. RANK, another member of the TNF-receptor family, has been identified on dendritic cells, and acts as the receptor for TRANCE/RANKL (Wong *et al*, 1997; Anderson *et al*, 1997).

Osteoprotegerin (OPG) is a soluble factor that belongs to the Tumour Necrosis Factor (TNF) receptor family. This factor is also known as Osteoclastogenesis Inhibitory Factor (OCIF). It has been shown to bind to TRANCE/RANKL/ODF, resulting in the inhibition of formation of functional multinucleate osteoclasts *in vitro*. OPG is a 401 amino acid polypeptide. Overexpression of OPG in transgenic mice results in severe osteopetrosis, with a loss of bone marrow cavities and profound depletion of osteoclasts. The same effects were observed upon administration of OPG to normal mice. Furthermore, OPG blocked ovariectomy-associated bone loss in rat. OPG mRNA

transcripts have been identified within bone and cartilage, vascular structures, midgut and kidney, and in several osteoblast cell lines. Current data suggest that OPG blocks the terminal stages of osteoclast differentiation, but not the formation of mononuclear osteoclast precursors (Simonet *et al*, 1997; Tsuda *et al*, 1997). The nomenclature adopted thoughout this specification is RANKL, OPG and RANK, in accordance with that proposed by Suda *et al.*, (1999).

The interaction between RANKL and OPG in the formation of osteoclasts is illustrated in Figure 1. Osteoclasts are derived from haematopoietic stem cells that differentiate along the monocyte/macrophage lineage. Mononuclear precursors of osteoclasts are required to come into direct or close contact with osteoblasts to be rendered capable of differentiating into mature, functional, multinucleate osteoclasts. Osteoblasts express RANKL, a membrane-bound protein that stimulates the differentiation and formation of multinucleate osteoclasts from mononuclear precursors when it binds to its receptor, RANK. RANKL expression is stimulated by bone-resorbing factors such as PTH, PGE_2, 1,25-dihydroxyvitamin D_3 and interleukins 6 and 11. The action of RANKL is antagonised by Osteoprotegerin, a soluble factor secreted by osteoblastic stromal cells. It binds to RANKL to inhibit the formation of differentiated multinucleate osteoclasts, but does not prevent the formation of mononuclear osteoclast precursors.

It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

We have now identified a polypeptide factor which is able to inhibit formation of mononuclear osteoclast precursors from haematopoietic stem cells, and which is expressed at least on the cell membranes of osteoblasts.

It appears that when the molecule is expressed on the osteoblast cell membrane it is not secreted. Preventing expression of the factor results in increased formation of mononuclear precursors of osteoclasts.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides a nucleic acid molecule which comprises a sequence encoding a protein which inhibits osteoclast differentiation from haematopoietic cell precursors, selected from the group consisting of osteoclast inhibitory lectin (OCIL) and OCIL-related protein

in which the nucleic acid molecule has a sequence selected from the group consisting of

(1) SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 44, SEQ ID NO: 45 or SEQ ID NO: 46

(2) a sequence able to hybridize to one or more of the sequences defined in (1) under conditions of moderate to high stringency; and

(3) a sequence having greater than 80% sequence identity with one or more of the sequences set out in (1).

Preferably the protein is expressed at least by osteoblasts.

Suitable conditions of moderate to high stringency are well known in the art. See the well-known textbook by Sambrook *et al* (1989), and Example 2 herein.

The nucleic acid may be cDNA, genomic DNA or messenger RNA. Preferably the nucleic acid molecule is a cDNA. More preferably the cDNA comprises a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 44, SEQ ID NO: 45 and SEQ ID NO: 46.

Preferably the protein inhibits differentiation of haematopoietic cells to osteoclast cells. In a particularly preferred embodiment, the nucleic acid molecule of the invention comprises a 110 base pair sequence as set out in SEQ ID NO: 2.

This aspect of the invention also encompasses anti-sense sequences directed against the nucleic acid molecule defined above, and particularly encompasses an anti-sense sequence directed against SEQ ID NO: 10. Preferably the anti-sense sequence is SEQ ID NO: 24 or SEQ ID NO: 25.

In a second aspect, the invention provides a polypeptide encoded by the nucleic acid molecule of the

invention. Preferably the polypeptide is encoded by the human cDNA sequence. More preferably the polypeptide comprises an amino acid sequence encoded by SEQ ID NO: 20.

In a third aspect, the invention provides an antibody directed against a polypeptide of the invention. Preferably the antibody is directed against an epitope present in a sequence selected from the group consisting of

Cys-Met-Ala-Gln-Glu-Ala-Gln-Leu-Ala-Arg-Phe-Asp-Asn-Gln-Asp-Glu-Leu-Asn-Phe (SEQ ID NO: 26),

Cys-Val-Thr-Lys-Ala-Ser-Leu-Pro-Met-Leu-Ser-Pro-Thr- Gly-Ser-Pro-Gln-Glu (SEQ ID NO: 48), and

Cys-Val-Gln-Lys-Pro-Glu-Glu-Gly-asn-Gly-Pro-Leu-Gly-Thr-Gly-Asp (SEQ ID NO: 49).

The antibody may be polyclonal or monoclonal, but is preferably monoclonal. Suitable methods for generating either polyclonal or monoclonal antibodies are very well known in the art. It will be clearly understood that the invention encompasses biologically-active fragments and analogues of such antibodies, including but not limited to ScFv fragments, trimeric antibodies, humanised antibodies and the like. Again, methods for producing such active fragments and analogues are well known in the art. See for example PCT/AU93/00491 and PCT/AU97/00212 and references cited therein.

In a fourth aspect, the invention provides a composition comprising a polypeptide or an antibody of the invention, together with a pharmaceutically-acceptable carrier.

Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, for example as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

In a fifth aspect, the invention provides a method of treatment of a condition characterised by abnormal bone resorption, comprising the step of administering an effective amount of a modulator of expression or function of the polypeptide of the invention.

Where the condition involves excessive bone resorption, the method will comprise administration of the polypeptide of the invention or the nucleic acid encoding this polypeptide, or a biologically-active fragment or analogue thereof. Such conditions include, but are not limited to, osteoporosis, primary hyperparathyroidism, Paget's disease, rheumatoid arthritis, renal osteodystrophy, humoral hypercalcaemia of malignancy, and conditions where cancer has metastasised to bone.

Conditions characterised by deficient bone resorption include osteopetrosis. Antibodies directed against the polypeptide of the invention or anti-sense oligonucleotides directed against the nucleic acid of the invention may be used to inhibit the function of the polypeptide and thus to increase bone resorption.

It is also contemplated that the polypeptide of the invention may be used to promote healing of bone fractures, particularly in individuals where fracture healing is delayed or deficient. These include individuals suffering from osteoporosis or diabetes mellitus.

Factors which influence bone resorption, such as parathyroid hormone-related protein and RANKL, affect breast development by altering apoptosis of cells. The OCIL factor of the invention also appears to alter apoptosis of cells, and may therefore participate in breast and lymph node development, similarly to other agents which modulate bone resorption.

Thus in a sixth aspect, the invention provides a method of modulating breast and lymph node development, comprising the step of administering an effective amount of a modulator of expression or function of the polypeptide of the invention to a subject in need of such treatment.

In a seventhth aspect, the invention provides a diagnostic kit, comprising a reagent selected from the group consisting of a nucleic acid of the invention or a fragment thereof capable of hybridising to a nucleic acid of the invention; an anti-sense nucleic acid of the invention; a polypeptide of the invention, and an antibody of the invention. For example, diagnostic kits for use in methods such as polymerase chain reaction, fluorescent *in situ* hybridisation, immunoassay, and the like are contemplated. Where appropriate, the molecule of the invention which is used may be labelled with a detectable marker, such as a radioactive, fluorescent, chemiluminescent or enzymic marker. Such diagnostic kits are useful for detection of abnormalities in the structure, expression or control of the factor of the invention, which may lead to increased bone resorption and concomitant pathological manifestations. They are also useful for screening of candidate drugs to assess their ability to modulate expression or function of the polypeptide of the invention.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 summarises the factors and mechanisms involved in control of osteoclast differentiation and development, as understood before the date of the present invention.

Figure 2 shows detection by Northern blotting of a 780 base pair mRNA species using rOCIL323 (SEQ. ID NO: 2) as a probe in a variety of rat clonal osteoblast-like cell lines, which were either untreated or subjected to treatment with 10^{-6} M retinoic acid for 24 hours.

Figure 3 shows the results of Northern blot analysis of rat clonal osteoblast-like cell lines treated

with 10^{-6} M retinoic acid, using rOCIL402 (SEQ. ID NO: 4), a 402 base pair fragment obtained by screening of a rat ROS 17/2.8 cDNA library using the polymerase chain reaction. Similar results were obtained using rOCIL323 fragment as a probe.

Figure 4 shows the comparison of rOCIL323 and rOCIL402 probes in Northern blotting of mRNA from 1,25-dihydroxyvitamin D_3-treated rat UMR 106-06 cells. The results showed that both fragments detected the same species of mRNA.

Figure 5 shows the results of Northern blot analysis of UMR 201 mRNA using mOCIL 2kb (SEQ ID NO: 10), showing that this probe detected the same 780 bp species as rOCIL323 and rOCIL402.

Figure 6 shows the intron-exon structures of the mOCIL gene (SEQ ID NO: 10) and of the mOCILrP1 gene ((SEQ ID NO: 11).

Figure 7 summarises the homology between mOCIL, mOCILrP1 and mOCILrP2.

Figure 8 shows the deduced protein sequences corresponding to mOCIL (Figure 8a), mOCILrP1 (Figure 8b), and mOCILrP2 (Figure 8c), illustrating the domain structure of each protein.

Figure 9 shows a comparison between the deduced protein sequences of mOCIL, mOCILrP1, and mOCILrP2, as generated using the program Clustal W.

Figure 10 shows the results of treatment of cocultures of primary mouse calvarial osteoblasts and mouse bone marrow cells with an anti-sense oligonucleotide, 323 A/S (SEQ ID NO: 22) and 402 A/S (SEQ ID NO: 23), directed against the C-type lectin region of OCIL, antisense oligonucleotide, 474 A/S (SEQ ID NO: 25), which was directed against the sequence in the open reading frame but outside the C-type lectin region, antisense oligonucleotide, 439 A/S (SEQ ID NO: 24), which was directed against the sequence upstream of the open reading.

a: cocultures treated with anti-sense

oligonucleotide under basal conditions.

b-d: cocultures stimulated with anti-sense oligonucleotides 323 A/S (SEQ ID NO: 22), 402 A/S (SEQ ID NO: 23), 439 A/S (SEQ ID NO: 24) and 474 A/S (SEQ ID NO: 25), in the presence of 1,25-dihydroxyvitamin D_3 and PGE_2.

Figure 11 shows the results of Northern blot analysis of mRNA from UMR106 parental cells, demonstrating upregulation of expression of OCIL by retinoic acid, PTH, IL-1α, IL-1β, IL-11, IL-17, TNFα, TGFβ, M-CSF, GM-CSF, PGE_2, 1,25-dihydroxy-vitamin D_3, 1,25-dihydroxyvitamin D_3 plus PGE_2, and PGE_2 plus dexamethasone.

Figure 12A shows the results of a time-course study, showing upregulation of OCIL by PTHrP.

Figure 12B shows that the upregulation could be detected using either rOCIL402 or mOCIL 2kb as the probe.

Figure 13 shows upregulation of expression of OCIL in primary mouse calvarial osteoblasts by IL-1α, IL-1β, IL-11, dexamethasone, and 1,25-dihydroxyvitamin D_3.

Figure 14A shows upregulation of expression of OCIL in ST2 mouse stromal cells by PGE_2, dexamethasone, 1,25-dihydroxyvitamin D_3, IL-11, PTH, and 1,25-dihydroxy-vitamin D_3 plus PGE_2.

Figure 14B shows the time course of upregulation of OCIL expression in ST2 cells by dexamethasone.

Figure 15 shows the constitutive expression of mOCIL and mOCILrP1/rP2 mRNA during osteoclast formation in mouse bone marrow cell cultures.

Figure 16 shows the results of Northern blot analysis of adult mouse tissues (left panel) and adult rat tissues (right panel), demonstrating expression of OCIL.

Figure 17 shows a schematic comparison between the sequences of rOCIL402, rOCIL1.3kb, rOCIL323 and rOCIL, illustrating differences between the 3' and 5' end regions.

Figure 18a compares the exon structures of four different hOCIL clones.

Figure 18b shows the deduced protein sequences corresponding to hOCIL clone 3, illustrating the domain

structure of the protein.

Figure 19 is a schematic representation of plasmid constructs used for recombinant expression of soluble mOCIL (Figure 19a) and soluble rOCIL (Figure 19b).

Figure 20 shows the effect of recombinant rOCIL (Figure 20a) protein or mOCIL protein (Figure 20b) on formation of multinucleate osteoclasts from mouse calvarial osteoblast-spleen cell cocultures.

Figure 21 shows the effects of hM-CSF and sRANKL in the absence or presence of mOCIL protein on osteoclast formation in mouse spleen cell cultures.

Figure 22 is a schematic representation of a plasmid construct used for recombinant expression of an MBP-mOCIL fusion protein.

Figure 23 is a schematic representation of a plasmid construct used for recombinant expression of an MBP-mOCILrP1 fusion protein.

Figure 24 is a schematic representation of a plasmid construct used for recombinant expression of an MBP-mOCILrP2 fusion protein.

Figure 25 is a schematic representation of a plasmid construct used for recombinant expression of an MBP-hOCIL fusion protein.

Figure 26 shows the effects of hM-CSF and sRANKL in the absence or presence of MBP or MBP-mOCIL fusion protein on osteoclast formation in total spleen cell (Figure 26a) or T cell-depleted mouse spleen cell cultures (Figure 26b).

Figure 27 shows the effects of hM-CSF and sRANKL in the absence or presence of the fusion proteins MBP-mOCILrP1 (Figure 27a) and MBP-mOCILrP2 (Figure 27b) on osteoclast formation in T cell-depleted mouse spleen cell cultures.

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of example only, with reference to the following non-

limiting examples and drawings.

We set out to clone a gene encoding a peptide that would function to prevent osteoclast formation. It is known that mature osteoblasts have limited potential to support osteoclast formation, and we postulated that mature osteoblasts might express osteoclastogenic inhibitors. The pre-osteoblastic cell line UMR201 can be differentiated to a more mature osteoblastic phenotype by treatment with 10^{-6} M retinoic acid for 24 hr (Ng *et al*, 1988). mRNA species differentially expressed between mature osteoblasts (retinoic acid-treated UMR201 cells) and immature osteoblasts (untreated UMR201 cells) were identified using an array of oligonucleotide primers in reverse transcription PCR, where products amplified from RNA from the two cellular populations were compared. We characterised products which were elevated in mature osteoblasts as candidates for osteoclastogenic inhibitory molecules.

Abbreviations used herein are as follows:

GM-CSF	granulocyte/macrophage colony stimulating factor
hPTH	human parathyroid hormone
IGF	insulin like growth factor
IL	interleukin
LIF	leukaemia inhibitory factor
M-CSF	macrophage colony stimulating factor (CSF-1)
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
OCIF	osteoclastogenesis inhibitory factor
ODF	osteoclast differentiation factor
OPG	osteoprotegerin
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
TGF	transforming growth factor

TNF	tumour necrosis factor
TRAP	tartrate-resistant acid phosphatase.

Throughout this specification amino acids are represented using the conventional single-letter code.

Example 1: Isolation of Rat cDNA Encoding the Inhibitory Factor

Total RNA was isolated from retinoic acid-treated preosteoblastic UMR201 cells using guanidine thiocyanate (Chomczynski *et al*, 1987). First strand cDNA was synthesised from 2 µg of total RNA by incubating for 1 h at 42°C with 15 units of AMV reverse transcriptase (Promega, Madison, WI) following oligo priming with the 3'adaptor primer 5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3') (Clontech, California, USA). A sense primer that was complementary to rat calcitonin cDNA, designated primer CT1:

CT1 5'-ATG CTG GGC ACG TAC ACA CAA-3' (SEQ ID NO:1)

and 3'UAP 5'- GGC CAC GCG TCG ACT AGT AC-3' (Clontech, California, USA) were used as primers in the polymerase chain reaction (PCR). The PCR conditions utilised a touchup PCR protocol with denaturation at 94°C for 5 min, and then 5 cycles at 94°C for 1 min, 37°C for 1 min and 72°C for 1 min, followed by 35 cycles of 94°C for 1 min, 49°C for 1 min and 72°C for 1 min. For these experiments, Expand High Fidelity PCR System (Boehringer Mannheim) was used in a Perkin Elmer Cetus 480 thermal cycler. A 321 bp PCR product was obtained. This 321 bp fragment, which we designated rOCIL323 (SEQ ID NO: 2), was used as a probe in Northern blots. As shown in Figure 2, it hybridised to a 780 bp mRNA species in UMR 201, UMR 201-10B, UMR 106-06, UMR 106-01 and ROS 17/2.8 cells, all of which are rat clonal osteoblast-like cell lines.

Since retinoic acid and PTH enhance OCIL mRNA expression dramatically in UMR 106-06 and UMR 106 parental cells, a similar RT-PCR procedure was carried out using RNA

isolated from retinoic acid or hPTH 1-34 treated UMR 106 parental cells. A PCR product identical to the 321 bp fragment for rOCIL323 was obtained, and its expression was found to be upregulated in UMR 106 cells treated with either retinoic acid or hPTH 1-34.

To extend the sequence of OCIL, anchored PCR was used to screen a rat ROS 17/2.8 cDNA library with λgt11 arms. An antisense 25 bp primer, designated OCILr1:

OCILr1 5'-TGA GTG TTG TCT GTC CAC TTC CAA G-3' (SEQ ID NO: 3)

complementary to a sequence in the 321 bp fragment, was used with either the λgt11 forward primer 5'-GGT GGC GAC GAC TCC TGG AGC C-3' or λgt11 reverse primer 5'-GAC ACC AGA CCA ACT GGT AAT G-3' (Clontech) to amplify an aliquot (10^6 plaque forming units) of the recombinant library. Cycling parameters were 94°C for 5 min, then 80 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, followed by a final extension step of 72°C for 10 min. A 402 bp fragment was obtained with λgt11 reverse primer as the anchored primer. Sequencing of this 402 bp fragment showed 88.6% identity over a length of 97 bp with rOCIL323 (SEQ ID NO: 2). The 402 bp fragment, designated rOCIL402, whose sequence is set out in SEQ ID NO: 4, was used to probe Northern blots obtained from the rat osteoblast-like cell line. It hybridised to the same 780 bp mRNA species observed with the rOCIL323 probe. These results are shown in Figures 3 and 4. The same results were obtained in both the presence and absence of 1,25-dihydroxyvitamin D_3.

A 3' Rapid Amplification of cDNA Ends (3'-RACE) strategy was used to obtain the 3' ends of the cDNA of interest. First-strand cDNA was synthesised from total RNA isolated from hPTH 1-34 treated UMR 106 parental cells by incubating for 1 h at 42°C with 15 units of AMV reverse transcriptase (Promega, Madison, WI) following priming with

the 3'adaptor primer 5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3') (Clontech, California, USA) according to the manufacturer's instructions. The sense specific primers used were OCILr11 (SEQ ID NO: 5)

OCILr11 5'-GAA ACA TCC CCC TGG AGT ATC C-3'

and OCILr12 (SEQ ID NO: 6)

OCILr12 5'-CCA AGT AAC TGG ACA TTG AGC CAG A-3'

complementary to sequences within rOCIL402 (SEQ ID NO: 4). First-strand cDNA was synthesised from total RNA isolated from hPTH 1-34 treated UMR 106 parental cells, using the oligo dT-anchor primer. The cDNA was further amplified by PCR using OCILr11 or OCILr12 and 3'UAP 5'-GGC CAC GCG TCG ACT AGT AC-3' (Clontech, California, USA) PCRs were run at 94°C for 5 min, then 35 cycles of 94°C for 30 s, 62°C for 30s, and 72°C for 2 min, followed by a final extension step of 72°C for 10 min. Three different polyadenylated 3' sequences were obtained, designated rOCIL1.3kb (SEQ ID NO: 7), rOCIL738bp (SEQ ID NO: 8) and rOCIL620bp (SEQ ID NO: 9) respectively. The region of sequence identity between rOCIL323 and rOCIL402 was found to extend to 117 bp.

Example 2: Isolation of Mouse cDNA and gDNA Encoding the Inhibitory Factor

rOCIL402 was labelled with [^{32}P] α-dCTP by using the Random Primer labelling kit (Boehringer Mannheim), and a mouse liver cDNA library was subjected to hybridisation screening at 65°C in a hybridisation buffer containing 4 x SSPE (SSPE contains 0.15 M NaCl, 0.01 M NaH_2PO_4, and 0.001 M EDTA), 5 x Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) for 24 hr. The filters were then washed sequentially in 2 x SSC at 65°C for 15 min, 2 x SSC with 0.1% SDS at 65°C for 30 min, and finally 0.1 x SSC at 65°C

for 10min. We obtained a 1907bp mouse cDNA, designated mOCIL2kb (SEQ ID NO: 10). The sequence of mOCIL2kb shows 80% identity over a length of 461 bp to that of rOCIL1.3kb. When used as an antisense riboprobe in Northern blot analysis, mOCIL2kb hybridised to a 780 bp mRNA species in UMR201 as detected by rOCIL323 and rOCIL402,as shown in Figure 5.

A cDNA fragment corresponding to the nucleotides 58-776 of mOCIL2kb was used as a cDNA probe to screen a genomic BAC Mouse I Hybridisation library. The screening was performed under contract by Genome Systems, Inc. According to their protocol, the cDNA fragment was labelled with [^{32}P] α-dCTP by random primer labelling and the library was screened under the hybridisation conditions of 55°C in a hybridisation buffer containing 5.5 x SSC, 5 x Denhardt's solution, 0.5% SDS and 0.5 x HEPES buffer for 18 hr. The filters were then washed sequentially in 1mM Tris-HCl pH 8.0 and 1% sarkosyl for 15 min, and 3 times in 1mM Tris-HCl pH 8.0 for 15 min washes at room temperature. Eight positive clones were isolated, of which seven positive clones were screened. After the genomic DNA was digested with HindIII or BamHI, Southern blot analyses were carried on with the same cDNA probe. Four clones (db.20147, db.20149, db.20151 and db.20152) were related, and other clones (db.20150, db.20153 and db.20154) are yet to be extensively analysed. The clones db.20147 and db.20149 were identical, and these differed from the other two identical clones db. 20151 and db.20152. These four clones were sequenced by subcloning into the pBS vector and by direct sequencing of the genomic clone using cycle sequencing. Sequencing of 8622 bp of the genomic clone (db.20152) of mOCIL was completed (SEQ ID NO: 37). It contains 5 exons, as shown in Figure 6. The 5' flanking region adjacent to exon I contains an A/T-rich motif, AATAAA, as TATA box gene promoter. Sequencing of 9862 bp of the genomic clone (db. 20149) was also completed (SEQ ID NO: 11). It contains 6 exons, as shown in Figure 6. The

sequences of exons I and II were completely different to that of mOCIL exon 1. The sequence of exons III to VI was 90.28% identical to that of mOCIL from exon II. The 5□ flanking region adjacent to exon I is a GC-rich region, containing a Sp 1 binding site. In combination, these features indicated that in fact this was a different gene. A search of the GenBank database showed that exons I and II showed 100% identity to a cDNA clone encoding a C-type lectin expressed in mouse bone marrow-derived dendritic cells, which was deposited in the GenBank data base on 20 January 1999 (Accession No. AF121352) and released on 15 June 1999. However, exons III to VI are 92% identical to AF121352 cDNA. The sequence of this genomic clone is redesignated as mOCIL-related protein 1 (mOCILrP1) gene (SEQ ID NO:11). The full length mOCILrP1 cDNA was originally thought to be a splice variant of mOCIL, and is 990 bp in length (SEQ ID NO: 12).

To confirm the mOCIL (SEQ ID NO: 36) and mOCILrP1 (SEQ ID NO:12) cDNA sequences, RT-PCR was carried out using total RNA isolated from ST2 mouse stromal cells, primary mouse calvarial osteoblasts and mouse liver tissue. The sense primer represented nucleotides 18-36 of mOCILrP1 (SEQ ID NO:12), and is designated as primer OCILm47 (SEQ ID NO: 13),

OCILm47 5'- TCC CAT GCC AGA TTG CTT G-3'

The antisense primer, which was originally designed from mOCIL2kb (SEQ ID NO: 10) nucleotides 136-157, represented nucleotides 746-725 of mOCIL (SEQ ID NO: 36) and is designated primer OCILm12 (SEQ ID NO: 14),

OCILm12 5'-GGG ACC ATA GGG GAA AGA GTA G-3'

The PCR was run at 94°C for 5 min, then 35 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. Seven clones

containing a 721 bp fragment were obtained from three sources, ST2 mouse stromal cells, primary mouse calvarial osteoblasts and mouse liver tissue. In 2 of the 7 clones, there was 100% identity to mOCILrP1 sequence, and 92.2% identity to mOCIL after the first 115 bp. In the other 5 clones, when compared to the mOCILrP1 sequence, there was 100% identity in the first 106 bp (exons I and II), but only 90.5% identity in the remaining 615 bp. This 721 bp fragment, originally designated as mOCIL47, was redesignated as mOCILrP2 (SEQ ID NO: 15). MOCILrP2 is related to, but distinct from, mOCIL (SEQ ID NO: 36) and mOCILrP1 (SEQ ID NO: 12).

A sense primer representing nucleotides 343-364 of mOCIL2kb (SEQ ID NO:10) and representing nucleotides 34-57 of mOCIL (SEQ ID NO:36), designated as OCILm17 (SEQ ID NO: 16),

OCILm17 5□-TGG AAA CTC AGC TCC TCA GCT CTG-3□

and antisense primer OCILm12 were also used to carry out RT-PCR with RNA from three sources, ST2 mouse stromal cells, primary mouse calvarial osteoblasts and mouse liver tissue, as above. PCR was run under the same conditions as above. Ten clones were obtained, each containing a 713 bp fragment. This sequence is designated mOCIL17 (SEQ ID NO: 17), and is 100% identical to mOCIL (SEQ ID NO: 36).

RT-PCR was also carried out using a sense primer corresponding to the region located at the junction of exons II and III, representing nucleotides 245-269 of mOCIL (SEQ ID NO: 36) and at the junction of exon III and exon IV, representing nucleotides 243-267 of mOCILrP1 (SEQ ID NO: 12), and designated primer OCILm32 (SEQ ID NO: 18),

OCILm32 5'- TTT GTC AGC AAC AAA GAC AGA ACA G-3'

The primer oligonucleotide OCILm32 has 24 of 25 bp complementary to mOCILrP1:

5'-TTTGTCAGCAACAAAGACAGAACAG-3' Primer
||||||| ||||||||||||||||
3'-AAACAGTCATTGTTTCTGTCTTGTC-5' mOCILrP1 (267) Strand -

Primer OCILm12 was used as an antisense primer. RT-PCR was carried out with RNA from three sources, ST2 mouse stromal cells, primary mouse calvarial osteoblasts and mouse liver tissue, as above. PCR was run under the same conditions. Four clones were obtained, each containing a 502 bp fragment. Three of the four clones have 100% identity to mOCIL (SEQ ID NO: 36) and one of the four clones is 100% identical to mOCILrP1 (SEQ ID NO: 12).

RT-PCR was also carried out using the sense primer OCILm47 (SEQ ID NO: 13) and an antisense primer representing nucleotides 855-874 of mOCILrP1 (SEQ ID NO: 12), designated primer OCILm49 (SEQ ID NO: 38),

OCILm49 5'-GTG GTT GCT CAG ATG TGA AC-3'

RT-PCR was carried out with RNA from the same three sources, ST2 mouse stromal cells, primary mouse calvarial osteoblasts and mouse liver tissue, as above. PCR was run under the same conditions. Two clones were obtained, each containing a 856 bp fragment with 100% identity to AF121352 and in which the first 721 bp are 100% identical to mOCILrP2 (SEQ ID NO: 15).

To further confirm that mOCILrp2 is AF121352, an antisense primer was designed based on the sequence of AF121352 (nucleotides 908-929), designated as primer OCILm48 (SEQ ID NO: 39),

OCILm48 5'-TTC ACA CAT CCC AGA AGA GGA C-3'

OCILm47 (SEQ ID NO: 13) was used as sense primer. RT-PCR was carried out under same conditions as above. Two clones were obtained, each containing a 916 bp fragment which has

100% identity to AF121352 and in which the first 721 bp is 100% identical to mOCILrP2 (SEQ ID NO: 15).

The full length mOCILrP2 cDNA is 988 bp in length. Its first 123 bp is 100% identical to mOCILrP1, but only 91.7% identical in the remaining 865 bp. Figure 7 summarises the homology between mOCIL, mOCILrP1 and mOCILrP2. The three different sequences (SEQ ID NO: 12, 15 and 36), which overall have 87% identity, may represent gene duplications, where either one or all three sequences may have similar biological outcomes. The functional data we have to date, relating to the inhibition of osteoclast formation from haemopoietic precursor cells using antisense oligonucleotides (SEQ ID NO: 24 and 25), have been obtained mainly with mOCIL17 (SEQ ID NO: 17), although experiments with recombinant protein (see below) indicate that the extracellular domains of mOCIL, mOCILrP1 and mOCILrP2 respectively can inhibit osteoclast formation.

mOCIL has an open reading frame encoding a 207 amino acid protein. As shown in Figure 8a, its putative protein structure is typical of a type II membrane protein, with a predicted 143 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 43 amino acid cytoplasmic domain. The extracellular domain has 5 cysteine residues. There are three potential N-linked glycosylation sites at residues 74, 100 and 158, all of which are in the extracellular domain. The putative protein sequence for mOCIL is designated mOCIL protein (SEQ ID NO: 40).

Comparison of the putative protein sequences derived from the rOCIL323, rOCIL1.3kb and mOCIL cDNA sequences with the SwissProt protein database indicated that the mOCIL protein sequence included a 113 amino acid C-lectin type motif, from positions 80 to 192 in the mOCIL protein sequence (SEQ ID NO: 40). This C-lectin motif is similar to that of CD69, a membrane-bound lectin expressed by bone marrow haematopoietic cells, and thought to be involved in monocyte differentiation. C-lectin motifs are

also involved in cell-cell contact and lipid binding (Sharon and Lis, 1995; Gabius 1997; Kieda, 1998).

mOCILrP1 has an open reading frame encoding a 218 amino acid protein. The putative protein sequence for mOCILrP1 is designated mOCILrP1 protein (SEQ ID NO: 41). Its structure is also typical of a type II membrane protein, with a predicted 142 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 55 amino acid cytoplasmic domain. The mOCILrP1 protein sequence also has a 113 amino acid C-lectin type motif, from positions 92 to 204 in the mOCILrP1 protein sequence (Figure 8b). The extracellular domain has 6 cysteine residues. There are three potential N-linked glycosylation sites at residues 86, 112 and 207, all of which are in the extracellular domain. There is a myristylation motif in the intracellular domain.

MOCILrP2 has an open reading frame encoding a 217 amino acid protein. The putative protein sequence for mOCILrP2 is designated mOCILrP2 protein (SEQ ID NO: 42). Its structure is also that of a type II membrane protein, with a predicted 141 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 55 amino acid cytoplasmic domain. Similarly to mOCIL and mOCILrP1, the mOCILrP2 protein sequence has an 113 amino acid C-lectin type motif, from positions 92 to 204 in the mOCILrP2 protein sequence (Figure 8c). The extracellular domain has 5 cysteine residues. There are four potential N-linked glycosylation sites at residues 86, 95, 112 and 165, all of which are in the extracellular domain.

The three different mouse protein sequences (SEQ ID NO: 40, 41 and 42) overall have 89% identity as shown in Figure 9. There are differences in the intracelullar domains between mOCIL and mOCIL-related proteins, and these domains may have different functional roles. If the C-type lectins act as receptors, the intracellular domains may confer different properties as a result of signal transduction. Comparison of the protein sequences in the

intracellular domain against the PROSITE database, using the ScanProsite program, showed that mOCIL protein has a Casein Kinase II (CK2) phosphorylation site at position 16-19 in SEQ ID NO: 40. In contrast, mOCILrP1 protein has two Protein Kinase C (PKC) phosphorylation sites at positions 42-44 and 51-53 (SEQ ID NO: 41), while mOCILrP2 (SEQ ID NO: 42) has no phosphorylation sites.

The three different proteins, mOCIL, mOCILrP1 and mOCILrP2, may be distinguished by several criteria:

(a) Nucleotide sequence: mOCIL, mOCILrP1 and mOCILrP2 appear to be derived from a common ancestral gene; however there are nucleotide differences which permit identification of the three molecules using specific oligonucleotide primers in RT-PCR, as described in Example 5, Figure 15.

(b) Gene structure: The promoter of mOCIL is a TATA promoter (SEQ. ID. No. 37), while the promoter for mOCILrP1 is a GC-rich region containing a SP 1 binding site (SEQ. ID. No. 11).

(c) mOCIL expression is regulated by PTH, while the expression of mOCILrP1 and mOCILrP2 is not (see example 5).

(d) The polypeptide products of mOCIL, mOCILrP1 and mOCILrP2 can be distinguished using antibodies directed against peptide fragments of mOCIL (SEQ. ID. No. 48) and mOCILrP1/rP2 (SEQ. ID. No. 49) based on the intracellular domains of the respective proteins. These have been used in tissue localisation studies, as described in Example 6.

Example 3: *Isolation of Human cDNA Encoding the Inhibitory Factor*

[^{32}P] α-dCTP labelled rOCIL402 was used to probe a human fetal cDNA library under low stringency hybridisation conditions at 55°C in a hybridisation buffer containing 4 x SSPE, 5 x Denhardt's solution, 0.5% SDS for 24 hr, and then washed with low stringency at 2 x SSC with 0.1% SDS at 40°C for 15 min, and 1 x SSC/0.1% SDS at 40°C

for 15min. Eight positive clones were obtained after tertiary screening. Clone No. 6 is a 1.3 kb cDNA segment, whose sequence was designated hOCIL clone 6 (SEQ ID NO: 19). The putative protein sequence encoded by bp883-1059 was a C-type lectin moiety, which showed 73% homology to the C-type lectin sequence previously demonstrated in rOCIL323, rOCIL1.3kb and mOCIL2kb. However, regions of amino acid sequence 5' and 3' to this C-type lectin domain were different from those of the mouse and rat sequences, as shown in Figure 6.

Clone No. 8 is 960 bp long. It has 64% identity over a length of 145 bp with rOCIL402. A search of the EST database showed that clone No. 8 has 99.5% sequence identity with an EST clone of unknown function from human pregnant uterus, Accession No. AA029932, over the published length of this EST 209 bp. This EST clone was ordered and further sequenced. The EST clone is 680 bp in length, and has 64% identity with rOCIL1.3kb over a length of 343 bp. It also has 64% identity over a length of 346 bp compared to mOCIL. RT-PCR showed that clone No 8 and AA029932 represent overlapping clones, which are contiguous, and combine to represent a human OCIL clone 1 of 1305 bp in length (SEQ ID NO: 20).

The deduced protein sequence has 56% homology to the deduced protein sequence of rOCIL1.3kb, and 62% homology to that of mOCIL. These differences are principally at the N-terminus. Although there is 80% homology between the mouse and human OCIL proteins in certain regions, this indicates that the mouse cDNA could not reliably be used to isolate a human genomic DNA encoding hOCIL.

In order to obtain the hOCIL gene, the 680 bp cDNA insert of clone AA029932 was isolated and screened by Genome Systems, Inc. against the genomic BAC Human Release II Hybridisation library, as described in Example 2. One positive clone was obtained. This genomic sequence, corresponding to the sequence from 654bp-1304bp of hOCIL,

clone has 100% identity to a sequence segment within a human genomic clone of 178,607 bp, which was deposited in the GenBank database on April 6, 1999 (Accession No. AC007068), and the 5' flanking region, promoter region and the first 654 bp of cDNA sequences are represented in the 74,801 bp sequence deposited in the GenBank database on December 9, 1999 (Accession No. AC010186).

The hOCIL gene is located in chromosome 12p. Chromosome 12 and chromosome 11 are considered to be evolutionarily related. There are several examples of evolutionarily related proteins whose genes are located on chromosome 12 and chromosome 11, such as PTH and PTHrP, IGF and IGF I , Harvey ras sarcoma 1, and Kisten ras sarcoma 2, etc. (Martin et al., 1991). Thus chromosome 11 and chromosome 12 share genes of similar biological characteristics with redundant function.

Example 4: *Effect of Anti-Sense Oligonucleotides on Osteoclast Formation*

Primary mouse calvarial osteoblasts were cocultured with mouse bone marrow cells to generate mononuclear and multinucleate osteoclasts. Staining for tartrate-resistant acid phosphatase (TRAP), performed using a commercial leukocyte acid phosphatase kit from Sigma Diagnostics (St. Louis, MO, USA; Katsogiannis *et al*, 1998), identified these cells as osteoclasts. Under normal conditions, multinucleate functional osteoclasts are not formed unless the cocultures are stimulated with 1,25-dihydroxyvitamin D_3 and PGE_2.

Experiments were carried out to block translation of OCIL mRNA in order to determine the function of its translated product. Antisense oligonucleotides may also down-regulate mRNA levels, and thus may effectively decrease transcription as well as translation. Primary mouse calvarial osteoblasts were treated with antisense oligonucleotides. Four antisense oligonucleotide sequences were designed. Two of these antisense oligonucleotide

sequences were complementary to the C-type lectin region, and designated 323 (SEQ ID NO: 22) and 402 (SEQ ID NO: 23) respectively:

323 5'-GAG TGT TGT CTG TCC ACT TCC-3'

402 5'-TTT CCA ACT CCA ATC CAG TTT-3'

The 323 antisense oligonucleotide has 19 of 21 bp complementary to mOCILrP2 (SEQ ID NO: 15)

5'-GAGTGTTGTCTGTCCACTTCC-3' 323 antisense (SEQ ID NO: 22)
|||||||| ||||||||||
3'-GTCACAACAAACAGGTGAAGG-5' mOCILrP2 Strand +

and has 20 of 21 bp complementary to mOCILrP1 (SEQ ID NO: 12)

5'-GAGTGTTGTCTGTCCACTTCC-3' 323 antisense (SEQ ID NO: 22)
||||||||||||||||||||
3'-GTCACAACAGACAGGTGAAGG-5' mOCILrP1 Strand +

and 100% to mOCIL (SEQ ID NO: 36)

5'-GAGTGTTGTCTGTCCACTTCC-3' 323 antisense (SEQ ID NO: 22)
||||||||||||||||||||
3'-GTCACAACAGACAGGTGAAGG-5' mOCIL Strand +

The 402 antisense oligonucleotide (SEQ ID NO: 23) has 100% complementarity to mOCILrP1 (SEQ ID NO: 12) and has 20 of 21 bp complementary to mOCILrP2 (SEQ ID NO: 15) and mOCIL (SEQ ID NO: 36):

5'-TTTCCAACTCCAATCCAGTTT-3' 402 antisense (SEQ ID NO: 23)
||||||||||| ||||||||
3'-AAAGGTTGAGGTCAGGTCAAA-5' mOCILrP2 Strand +

5'-TTTCCAACTCCAATCCAGTTT-3' 402 antisense (SEQ ID NO: 23)
||| ||||||||||||||||
3'-AAAAGTTGAGGTTAGGTCAAA-5' mOCIL Strand +

The other two antisense oligonucleotide sequences, respectively designated 439 (SEQ ID NO: 24) and 474 (SEQ ID NO: 25), specifically inhibit the translation of mOCIL (SEQ ID NO: 36) but not mOCILrP1 (SEQ ID NO: 12) and mOCILrP2 (SEQ ID NO: 15).

The oligonucleotide 439 (SEQ ID NO: 24) is antisense to the sense primer OCILm17 and located upstream of the open reading frame:

439 5' GAG GAG CTG AGT TTC CAC TAC-3'

The antisense oligonucleotide 474 (SEQ ID NO: 25) is complementary to a region in mOCIL17 (SEQ ID NO: 17) in the open reading frame in the intracellular domain but outside the C-type lectin region:

474 5'-GGT AGG GAA GCC TTT GTG AC-3'.

Under basal conditions, *ie.* in the absence of stimulation with 1,25-dihydroxyvitamin D_3 and PGE_2, there was a 3- to 5-fold increase in the number of mononucleate TRAP-positive cells in the cocultures treated with the 323 (SEQ ID NO: 22) and 474 (SEQ ID NO: 25) antisense oligonucleotides over the period from 3 to 7 days. With the 402 antisense oligonucleotide (SEQ ID NO: 23), a 4.5-fold increase in the formation of mononucleate TRAP positive cells was observed after 7 days treatment. Multinucleate TRAP-positive cells (323 (SEQ ID NO: 22); 4.5 ± 2, 474 (SEQ ID NO: 25); 4.25 ± 1.25) were also observed in cocultures treated with both 323 (SEQ ID NO: 22) and 474 (SEQ ID NO: 25) antisense oligonucleotides at a concentration of 5μM, whereas none were observed in the control. These experiments were performed three times, and

a representative result is shown in Figure 10a.

When the cocultures were stimulated with 1,25-dihydroxyvitamin D_3 and PGE_2, multinucleate TRAP-positive osteoclasts were formed after 7 days. Treatment with 5 μM 323 antisense oligonucleotide (SEQ ID NO: 22) resulted in a seven-fold increase in the number of multinucleate osteoclasts, as shown in Figure 10b.

Treatment with 10 μM 402 (SEQ ID NO: 23), 5 μM 439 (SEQ ID NO: 24) and 474 (SEQ ID NO: 25) antisense oligonucleotide resulted in a 2 to 3-fold increase in the formation of multinucleate osteoclasts after 7 days, as shown in Figures 10c and 10d.

These TRAP-positive cells were further characterized as osteoclasts by the presence of receptors for calcitonin, demonstrated using autoradiography and immunostaining, and by the ability of these cells to form resorption pits in bone slices.

Effects of mOCIL antisense oligonucleotides on the three phases of osteoclast formation were also investigated. Mouse bone marrow and primary osteoblastic cells were cocultured in the absence of 1,25-dihydroxyvitamin D_3 and PGE_2 for a 7 day culture period. 323 (SEQ ID NO: 22) and 474 (SEQ ID NO: 25) antisense oligonucleotides were added for the 3 phases of culture: the first phase (0-3 days), in which there is proliferation of osteoclast progenitors, the second phase (3-5 days) and the final phase (5-7 days), in which these cells differentiate into mature osteoclasts. TRAP-positive osteoclasts were counted. In order to examine the role of OCIL on the bone resorptive activity of mature osteoclastic cells, the cells were also cultured on dentine slices under the same culture conditions as above, and resorption pits formed on dentine slices were quantitated. The results are shown in Table 1, and indicate that the OCIL acted at an early stage in osteoclast formation.

Table 1

Effects of mOCIL Antisense Oligonucleotides on the Three Phases of Osteoclast Development

TIME	TREATMENT	MONO	MNC	PITS
7 days	control	3596 ± 511.5	0	10 ± 4.4
0-3 days	323	6880 ± 674 *	7.7 ± 5.3	34 ± 15
	474	6893 ± 429.6 **	8.7 ± 1.7 *	20 ± 2.5
3-5 days	323	2840 ± 197.6	0	14 ± 6
	474	3110 ± 334	4.3 ± 2	24 ± 2.5 *
5-7 days	323	4236 ± 518.6	0	10.6 ± 0.79
	474	3363 ± 139.8	0	3.3 ± 2
0-7 days	474	5223 ± 571 *	3.3 ± 1	20.3 ± 5.9

mono mononuclear osteoclast precursors

MNC multinucleate osteoclasts

pits resorption pits formed on dentine slices

*$p < 0.05$ vs. control

**$p < 0.01$ vs. control

Example 5: *Regulation of expression of OCIL mRNA*

The regulation of OCIL mRNA expression was examined in the UMR106 parental osteoblast-like cell line using rOCIL402 as a probe. As shown in Figure 11, expression of the mRNA was upregulated by retinoic acid (RA), parathyroid hormone (1-34), parathyroid hormone related protein (1-34), TNF-α, interleukin 1α (IL-1 α), IL-1β, IL-11, IL-17, GM-CSF, M-CSF, TGFβ, dexamethasone, 1,25-dihydroxyvitamin D_3 and prostaglandin E_2. A time course study, illustrated in Figure 12, showed that parathyroid hormone-related protein (1-34) increased levels of OCIL mRNA as early as 1 hour, peaking at 4 hours and maintaining the high level of expression until 48 hours.

As shown in Figure 13, in primary mouse calvarial osteoblasts, OCIL mRNA was upregulated by IL-1 α, IL-1β IL-11, 1,25-dihydroxyvitamin D_3 and retinoic acid. In ST2 mouse stromal cells, OCIL mRNA was upregulated by dexamethasone, 1,25- dihydroxyvitamin D_3 and IL-11. The time course also showed that dexamethasone increased OCIL mRNA at 1 hour, peaking at 2 hours and returning at basal level at 24 hours. These results are illustrated in Figures 14A and 14B, respectively.

Example 6: *OCIL mRNA expression during osteoclast formation in mouse marrow cultures*

OCIL mRNA expression during osteoclast formation in mouse marrow cultures was investigated by RT-PCR. The mouse bone marrow cells were prepared and cultured for 8 days in the presence of 1,25-dihydroxyvitamin D_3, as described by Ikegame et al (1995). At each time point, total RNA was isolated. RT-PCR was carried out using OCILm17 (SEQ ID NO: 16) and OCILm12 (SEQ ID NO: 14) as sense and antisense primers respectively. The PCR was run at 94°C for 5 min, then 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. RT-PCR was also carried out to investigate mOCILrP1/rP2 mRNA expression during such

cocultures. Primers specific for mOCILrP1/rP2, which distinguish these from mOCIL, were OCILm47 (SEQ ID NO: 13), a sense primer located on the intracelullar domain of mOCILrP1 and mOCILrP2, and OCILm12 (SEQ ID NO: 14) as an antisense primer. The PCR was run at 94°C for 5 min, then 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. Southern blot analysis was carried out as described by Zhou et al (1994); 20 µl of each PCR reaction mixture was run on a 2% agarose gel, transferred to nylon membranes, and the products authenticated by probing with an internal antisense strand oligonucleotide, OCILr1 (SEQ. ID NO: 3). OCILr1, which has 24 of 25 bp complementary to mOCIL and mOCILrP1 and 23 of 25 bp complementary to mOCILrP2, was labelled with digoxigenin-dUTP using a 3'-tailing kit (Boehringer Mannheim). Hybridisation was carried out with 2 pmol/ml labeled oligonucleotides in a buffer containing 5 x SSC, 0.02% SDS, 0.1% sarcosine and 100 ng/ml poly A, at 55°C for 14 h. Detection was by chemiluminescence using CDP-star (Boehringer Mannheim), according to the manufacturer's instructions.

For comparison with another osteoclast inhibitor, OPG mRNA expression was also investigated. A set of sense and antisense primers was used as described by Horwood *et al.* (1998), having nucleotide sequences represented by OPG-7 (5'-TGAGTGTGAGGAAGGGCGTTAC-3', nucleotides 405-426) and OPG-3 (5'-TTTCTCGTTCTCTCAATCTC-3', nucleotides 1021-1040), respectively. The PCR was run at 94°C for 5 min, then 35 cycles of 94°C for 30s, 57°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. Hybridisation was carried out using digoxigenin labeled internal sense strand oligonucleotide, OPG-1 (5'-ACCAAAGTGAATGCCGAG-3') under the same conditions described above. To ensure equal starting quantities of RNA in each sample, the reverse transcribed material was also amplified using oligonucleotide primers specific for rat GAPDH (39). A 414 bp fragment was amplified using a 5'-specific

oligonucleotide, GAPDH-4 (5'-CATGGAGAAGGCTGGGGCTC-3', representing nucleotides 306-325 of rat GAPDH) and a 3'-specific oligonucleotide, GAPDH-5 (5'-AACGGATACATTGGGGGTAG-3', representing nucleotides 701-720). Products were verified with a digoxigenin-labelled internal sense strand oligonucleotide, GAPDH-1 (5'-GCTGTGGGCAAGGTCATCCC-3', representing nucleotides 640-659) using the hybridisation conditions described above.

As shown in Figure 15, OCIL mRNA was constitutively expressed in fresh bone marrow cells at a high level. When cultures were stimulated by 1,25-dihydroxyvitamin D_3, a time-dependent decrease in OCIL mRNA relative to GAPDH mRNA occurred. In contrast, OPG mRNA was constitutively expressed at a low level in fresh bone marrow cells, and this level was increased by treatment with 1,25-dihydroxyvitamin D_3 after 4 days. This increased mRNA level was maintained for up to 8 days culture in the presence of 1,25-dihydroxyvitamin D_3. As reported by Romas *et al.* (1996), in this system, multinucleate osteoclast formation was observed after day 5, correlated with the decrease in OCIL mRNA expression, and an increase in mRNA for IL-11Rα as well as calcitonin receptor.

PTH (1-34) or PTHrP (1-34), which influence bone resorption, have been shown to induce osteoclast formation in the coculture of primary mouse calvarial osteoblasts and mouse bone marrow cells. To investigate whether PTH 1-34 regulates mRNA expression for mOCIL and the related proteins, mOCILrP1 and mOCILrP2, RT-PCR was carried out using RNA isolated from primary mouse calvarial osteoblast cells which were treated with 100ng/ml hPTH-(1-34) over a time course of 0.5 to 72 hours. OCILm17 (SEQ ID NO: 16) and OCILm12 (SEQ ID NO: 14) were used as sense and antisense primers, respectively, to determine mOCIL mRNA expression. Oligonucleotides OCILm47 (SEQ ID NO: 13) and OCILm12 (SEQ ID NO: 14) were used as sense and antisense primers, respectively, to determine mOCILrP1/rp2 mRNA expression. PCR and Southern blot analyses were carried

out under the same conditions as described above. The results showed that mOCIL mRNA expression was upregulated five-fold by PTH at 1 hour, peaking at 2 hours and returning to basal levels by 4 hours treatment: levels were unchanged over the remainder of the experiment (24 hours). In contrast, mOCILrP1/rP2 mRNA was not regulated by PTH. This indicates that mOCIL is differentially regulated, while mOCILrP1 and mOCILrP2 are not.

Example 7: Localization of OCIL mRNA

mRNA encoding OCIL was localised in fetal, newborn and adult mouse tissues by *in situ* hybridisation using the rOCIL 402 antisense probe, using a method described previously (Katsogiannis et al, 1997 and 1998). Plasmid cDNA was labelled with digoxigenin (DIG) using an RNA labelling kit (Boehringer-Mannheim, Mannheim GmbH, Germany). Hybridisation signals were detected by alkaline phosphatase staining with BCIP/NBT after incubation with an anti-digoxigenin antibody coupled to alkaline phosphatase. The mRNA is expressed in a range of tissues, as summarized in Table 2.

Table 2

Adult Rat Tissues Probed with rOCIL402

	Tissue	OCIL mRNA
Calvaria	Osteoblasts	+++
	Marrow hematopoietic cells	++
	Megakaryocytes	++
Kidney	Medulla (collecting tubules)	+++
	Outer cortex (collecting tubules only)	+
	Glomeruli (endothelial cells only weakly positive)	-ve
	Proximal/distal tubules	-ve
Lung	pneumocytes	++
	bronchial epithelium	++
Brain	Neurones in cerebral cortex, cerebellar cortex, hippocampus; choroid plexus	+++
Heart	Cardiac muscle	+++
Spleen	White pulp	+++
	Cortex	+
	Red pulp	-ve
Gut	Luminal epithelium	++
Liver	Hepatocytes	-ve

In situ hybridization was also carried out to detect OCIL mRNA localisation in adult murine tissue and human skin, using the same method, and the results are summarized in Table 3.

Table 3

Normal Murine Tissues Expressing OCIL mRNA

Tissues	Fetal (day 15)	Newborn (day 1)	Adult (5-8 weeks)
Extraskeletal tissues			
Brain	+++	+++	+++
Lung	++	+++	-
Heart	+++	+++	++
Kidney (collecting tubules)	++	+++	-
Small Intestine	+	+	-
Liver	+/(mk=+++)*	+	-
Skeletal muscle	+++	+++	++
Skin	+++	+++	++
Spleen	nd	nd	++
Skeletal tissues/cells			
Long bone			
chondrocytes	+++	++	+++
osteoblasts	na	+++	+++
osteoclasts	nd	++ or -	++ or -
perichondrium/ periosteum	++	+++	++
marrow/megakaryocytes	na	++	++
Calvarial bone			
osteoblasts	+++	+++	++
osteoclasts	++	++	++
periosteum	nd	++	++

(+) denotes weak signal
(++) denotes moderate signal
(+++) denotes strong signal
(-) denotes absence of signal
(na) not applicable

(nd) not determined.
*(mk) megakaryoblast of fetal liver.

OCIL mRNA localization in human skin probed with OCIL402 (SEQ ID NO: 4):

Epidermis (all layers) +++
**Basal layer slightly weaker signal

In Northern blot analyses of adult mouse tissues using mOCIL2kb (SEQ ID NO: 10) as the probe, OCIL mRNA was shown to be expressed in heart, skin, lung, liver, kidney, gut and brain. In adult rat, OCIL mRNA was found to be expressed in brain, bone, lung, liver, gut, kidney, mouse, skin and heart. These results are illustrated in Figure 16.

Since the nucleotides 900-1907 of mOCIL2kb (SEQ ID NO: 10) were not part of the mOCIL sequence, the Northern blot analysis was performed using mOCIL17 (SEQ ID NO: 17) as probe, which detected the same 780 bp species of mRNA. Northern blot analysis was also carried out using plasmid containing the nucleotides 900-1907 of mOCIL2kb (SEQ ID NO: 10) only as a probe. This probe failed to hybridize with any mRNA.

Example 8: Confirmation of full length sequences

(a) Rat OCIL

A 5'-Rapid Amplification of cDNA Ends (5'-RACE) strategy was used to obtain the 5' ends of the rOCIL cDNA, using the SMART RACE cDNA Amplification Kit (Clontech, California, USA). The antisense primer used was OCILr25 (SEQ ID NO: 32)

OCILr25 5'-CTC AGT GTT GTC TGT CCA CTT CCA AGG G-3'

complementary to sequences within rOCIL402 (SEQ ID NO: 4). First-strand cDNA was synthesised from total RNA isolated

from hPTH 1-34 treated UMR 106 parental cells according to the manufacturer's instructions. The cDNA was further amplified by PCR using OCILr25 and UNP primer as the 5' anchored primer. The PCR conditions utilised a touchdown PCR protocol with denaturation at 94°C for 1 min, then 5 cycles at 94°C for 30 sec, 72°C for 1 min, and then 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 1 min, followed by 50 cycles of 94°C for 30 s, 65°C for 30s, and 72°C for 1 min. An extension of 398 bp of 5'- sequence of rOCIL1.3kb (SEQ ID NO: 7) was obtained. The full length rat OCIL sequence is 1628 bp, designated rOCIL (SEQ ID NO: 33). Figure 17 summarises the sequence of 402 (SEQ ID NO: 4), rOCIL1.3 (SEQ ID NO:7), rOCIL323 and rOCIL (SEQ ID NO: 33).

(b) Mouse OCIL

A 5'-RACE strategy was used to confirm the mOCIL2kb sequence. The antisense primers used were OCILr25 (SEQ ID NO: 32), which was 100% identical to mOCIL2kb (SEQ ID NO: 10), and a specific primer OCILm75 (SEQ ID NO: 34).

OCILm75: 5'-CAG TTT TGC GGG CAA GCA GCA TAG-3'

complementary to sequences within mOCIL2kb (SEQ ID NO: 10). First-strand cDNA was synthesised from total RNA isolated from mouse spleen cells according to the manufacturer's instructions. The cDNA was further amplified by PCR using OCILr25 or OCILm75 and UNP as the 5' anchored primer, in a touchdown PCR protocol with denaturation at 94°C for 1 min, then 5 cycles at 94°C for 30 sec, 72°C for 1 min, and then 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 1 min, followed by 40 cycles of 94°C for 30 s, 65°C for 30s, and 72°C for 1 min.

A 3'-RACE strategy was also used to obtain the 3' ends of the mOCIL cDNA. The sense specific primer used was OCILm76 (SEQ ID NO: 35).

OCILm76 5'-AGG CAG CCC GCA GGA GGT AGA AG-3'

complementary to sequences within mOCIL2kb (SEQ ID NO: 10). First-strand cDNA was synthesised from total RNA isolated from mouse spleen cells according to the manufacturer's instructions. The cDNA was further amplified by PCR using OCILm76 and UNP primer as the 3' anchored primer in a touchdown PCR protocol with denaturation at 94°C for 1 min, then 5 cycles at 94°C for 30 sec, 72°C for 1 min, and then 5 cycles of 94°C 30 sec, 70°C for 30 sec and 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30s, and 72°C for 1 min.

A full length mOCIL cDNA sequence of 1206 bp was obtained and designated mOCIL (SEQ ID NO: 36). This sequence confirmed that nucleotides 1-320 of the original mOCIL2kb represented an inverted repeat of the 3' end of the sequence, and that nucleotides 900-1907 of mOCIL2kb were not part of the mOCIL sequence.

(c) Human OCIL

A 5'-RACE strategy was used to confirm the hOCIL sequence. The specific antisense primer used was OCILh1 (SEQ ID NO: 43),

OCILh1: 5'-CTC TGC TCA GCC CAA TCC AGT GAT CAG-3'

complementary to sequences within hOCIL clone 1 (SEQ ID NO: 20). According to the manufacturer's instructions, first-strand cDNA was synthesised using human placental total RNA, which is included in the SMART RACE cDNA Amplification Kit (Clontech, California, USA). The cDNA was further amplified by PCR using OCILh1 and UNP primer as the 5' anchored primer using a touchdown PCR protocol with denaturation at 94°C for 1 min, then 5 cycles at 94°C for 30 sec, 72°C for 1 min, and then 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 1 min, followed by 50 cycles of 94°C for 30 s, 65°C for 30s, and 72°C for 1 min.

Three different 5' end sequences were obtained, designated hOCIL clone 2 (SEQ ID NO: 44), hOCIL clone 3 (SEQ ID NO: 45) and hOCIL clone 4 (SEQ ID NO: 46).

The length of hOCIL clone 2 (SEQ ID NO: 44), hOCIL clone 3 (SEQ ID NO: 45) and hOCIL clone 4 (SEQ ID NO: 46) is 820, 937 and 845 bp, respectively. Minor differences in the nucleotide sequences were noted between hOCIL clones 1, 2, 3 and 4. These were:

1) At position 545 in human OCIL clone 1 a "C" was called and a "C" was at this equivalent position in clones 2 and 3, whilst in clone 4 (at position 117), a "G" was called.

2) At position 649 in human OCIL clone 1 a "T" was called, and a "T" was called at this position in human OCIL clone 4, whilst a "C" was called in the equivalent positions for clones 2 (at position 164) and 3 (at position 189).

3) At position 835 in human OCIL clone 1 and at equivalent positions for hOCIL clones 2 and 3, a "G" was called, whilst at an equivalent position for human OCIL clone 4 (at position 467), an "A" was called.

Of these clones, only hOCIL clone 3 predicted a protein sequence, while hOCIL clones 1, 2 and 4 did not possess a Kozak sequence predicting an initiating methionine residue. Analysis of the genomic structure of human OCIL (below) predicts that hOCIL clones 1 and 2 result from read-through into intron II, and therefore, represent prespliced mRNA species (Figure 18a). HOCIL clone 4 appears to represent an alternatively spliced mRNA transcript that does not encode a full length protein. HOCIL clone 3 predicts a protein of 191 amino acids which is a C-lectin type II membrane-bound protein. The protein is predicted to have an intracellular domain of 30 amino acids, transmembrane domain of 29 amino acids and an extracellular domain of 132 amino acids. Within the extracellular domain is a C-lectin domain of 112 amino acids (amino acids 75 to 186; Figure 18b). The sequence

from 13 bp to 845 bp of hOCIL clone 3 is identical to that from 7 bp to 850 bp of LLT1, a cDNA of 850 bp encoding a C-type lectin expressed by Natural Killer (NK) cells, T cells, and B cells (GenBank database Accession No. AF133299). The features of hOCIL clone 3 identify this as encoding the human equivalent of mouse and rat OCIL.

The hOCIL gene is 46.5kb in length (SEQ ID NO: 21). The hOCIL gene is composed of 6 exons, and may be alternatively spliced at the 5' end, as illustrated in Figure 18a. hOCIL clone 1 (SEQ ID NO: 20), as well as hOCIL clone 2 (SEQ ID NO: 44) contains exons III, IV,V and VI. hOCIL clone 3 (SEQ ID NO: 45) contains exons II, III, IV, V and VI. hOCIL clone 4 (SEQ ID NO: 46) contains exons I, III, IV, V and VI.

A 3-RACE strategy was also used to obtain the 3' ends of the cDNA using the SMART RACE cDNA Amplification Kit (Clontech, California, USA). The sense specific primer used was OCILh3'-1 (SEQ ID NO: 47)

OCILh3'-1 5'-GCTGATCTTGCTCAGGTTGAAAGCTTCC-3'

complementary to sequences within hOCIL (SEQ ID NO: 20). First-strand cDNA was synthesised from total RNA isolated from MG63 cells, a human osteosarcoma cell line, according to the manufacturer's instructions. The cDNA was further amplified by PCR using OCILh3'-1 and UNP primer as the 3' anchored primer. The PCR conditions utilised a touchdown PCR protocol with denaturation at 94°C for 1 min, then 5 cycles at 94°C for 30 sec, 72°C for 1 min, and then 5 cycles of 94°C 30 sec, 70°C for 30 sec and 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30s, and 72°C for 1 min.

3' RACE confirmed the 3'end sequence of the hOCIL clones.

Example 9: Antibodies Directed Against OCIL

The following peptide fragment of the deduced amino acid sequence derived from the cDNA sequence of mOCIL17 (SEQ ID NO: 17) was synthesized, and was used to immunize rabbits, using standard protocols.

Cys-Met-Ala-Gln-Glu-Ala-Gln-Leu-Ala-Arg-Phe-Asp-Asn-Gln-Asp-Glu-Leu-Asn-Phe (SEQ ID NO: 26)

This peptide sequence showed a high homology to mOCILrP1 (SEQ ID NO: 41) and mOCILrP2 (SEQ ID NO: 42), as follows:

```
1     CMAQEAQLARFDNQDELN
      ||||||||||||||||||
108   CMAQEAQLARFDNQDELN   mOCIL (SEQ ID NO: 40)

1     CMAQEAQLARFDNQDELN
      |||||||||||||  |||
120   CMAQEAQLARFDNEKELN   mOCILrP1 (SEQ ID NO: 41)

1     CMAQEAQLARFDNQDELN
      |||||||||||||  ||
120   CMAQEAQLARFDNEEELI   mOCILrP2 (SEQ ID NO: 42)
```

Two specific peptide fragments of the deduced amino acid sequence derived from the cDNA sequence of mOCIL (SEQ ID NO: 36) and mOCILrP1/mOCILrP2 (SEQ ID NO: 12 and 15) in the intracellular domain were synthesised, and were also used to raise antibodies:

Antibody MOCIL-3 is specific for an epitope in the following sequence of mOCIL:

Cys-Val-Thr-Lys-Ala-Ser-Leu-Pro-Met-Leu-Ser-Pro-Thr-Gly-Ser-Pro-Gln-Glu (SEQ ID NO: 48)

Antibody MOCIL-RP-1 is specific for an epitope in the following sequence of mOCILrP1/mOCILrP2:

Cys-Val-Gln-Lys-Pro-Glu-Glu-Gly-Asn-Gly-Pro-Leu-Gly-Thr-Gly-Asp (SEQ ID NO: 49)

The antibodies raised may be used to detect the OCIL protein, using standard immunohistochemical methods, or to neutralize OCIL activity in murine co-cultures to stimulate osteoclast formation.

Example 10: Immunohistochemistry

Rabbit polyclonal antibodies prepared as described in Example 7 were used for immunohistochemistry. A kit for the standard peroxidase-labelled streptavidin-biotin detection method (DAKO, Boenisch, 1989) was used according to the manufacturer's instructions, with minor modifications. The dilution of the antiserum used was optimised in preliminary experiments. Incubation of tissue sections with a 1:100 dilution of the primary antiserum was carried out overnight at 4°C in a humidified chamber. Peroxidase activity was detected with 3'-3'-diamino-benzidine tetrahydrochloride (Sigma) and 0.15% H_2O_2. Slides were counterstained with haematoxylin, dehydrated and mounted on a coverslip. The tissue expression of mOCIL, mOCILrP1 and mOCILrP2 proteins as detected using the 3 antibodies raised against the sequences SEQ ID NO: 26; SEQ ID NO: 48 and SEQ ID NO: 49 was identical. The results are summarized in Table 4.

Table 4

Normal Murine Tissues Expressing mOCIL or mOCILrP protein

Tissues	Fetal (day 15)	Newborn (day 1)	Adult (5-8 weeks)
Extraskeletal tissues			
Brain	nd	+	+++
Lung	nd	++	+
Heart	nd	++	++
Kidney (collecting tubules)	nd	++	++
Small Intestine	nd	nd	nd
Liver	nd	nd	nd
Skeletal muscle	nd	++	++
Skin	nd	++	++
Spleen	nd	nd	nd
Skeletal tissues/cells			
Long bone			
chondrocytes	nd	++	++
osteoblasts	na	+++	+++
osteoclasts	nd	nd	nd
perichondrium/periosteum	nd	++	++
marrow/megakaryocytes	na	++	++

(+) denotes weak signal;
(++) denotes moderate signal;
(+++) denotes strong signal;
(−) denotes absence of signal;
(na) not applicable;
(nd) not determined.

Example 11: Production of Recombinant OCIL protein in a mammalian expression system

OCIL proteins were prepared by recombinant DNA technology to allow more extensive laboratory studies of their actions on osteoclast formation as well as osteoblast function. Soluble mouse and rat OCIL cDNA tagged at the N-terminus with the FLAG epitope were constructed in the pEF-BOS Mammalian expression vector (Mizushima & Nagata 1990), which had been modified to contain an in-frame IL-3 signal sequence and FLAG peptide coding sequence (gift of Dr. D Hilton).

In order to obtain a RT-PCR product encoding the mOCIL (SEQ ID NO: 36) extracellular domain (amino acids 63-207) to clone into the MluI site of the vector, as shown in Figure 19a, the RT-PCR was carried out using total RNA isolated from primary mouse calvarial osteoblasts, which support osteoclast differentiation in coculture. A sense primer, OCILm33, comprising OCILm32 representing nucleotides 245-269 of mOCIL (SEQ ID NO: 36) and containing a MluI site, designated primer OCILm33 (SEQ ID NO: 27):

OCILm33 5'-GCC ACG CGT TTG TCA GCA ACA AAG ACA GAA CAG-3'

and an antisense primer representing nucleotides 746-725 of mOCIL (SEQ ID NO: 36) and containing a MluI site, designated primer OCILm46 (SEQ ID NO: 28),

OCILm46 5'-GCC ACG CGT GGG ACC ATA GGG GAA AAA GTA G-3'

were used as primers in the PCR. PCR was run at 94°C for 5 min, then 35 cycles of 94°C for 30 s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. A 501 bp fragment was obtained and further cloned into the expression vector pEF-BOS. Sequencing confirmed the open reading frame and FLAG fusion (bp 1-132) and the 501 bp fragment sequence (bp133-633) (SEQ ID NO: 29), which was confirmed to be identical to mOCIL17

(SEQ ID NO: 17). The sequencing results also showed that the primer OCILm46 had 22 of 23 bp complementary to mOCIL17 (SEQ ID NO: 17) and mOCIL (SEQ ID NO: 36):

5'-GCCACGCGTGGGACCATAGGGGAAAAAGTAG-3' Primer OCILm46
||||||||||||||||| |||||
3'-ATCGTGAAACCCTGGTATCCCCTTTCTCATC-5' mOCIL (725) Strand +

To obtain a PCR product encoding the rOCIL1.3kb (SEQ ID NO: 7) extracellular domain (amino acids 40-179), a sense primer to represent nucleotides 126-146 of rOCIL1.3kb with the MluI site, designated primer OCILr22 (SEQ ID NO: 30),

OCILr22 5'- GCC ACG CGT TCA GTA AAA AAG ACA GCC AAG-3'

and an antisense primer representing nucleotides 544-526 of rOCIL1.3kb with the MluI site, and designated primer OCILr23 (SEQ ID NO: 31),

OCILr23 5'-GCC CAG CGT AAC TAC AGG CAC TGT GAG G-3'

were used as primers in a PCR. PCR was carried using rOCIL1.3 kb plasmid as a template and run at 94°C for 5 min, then 35 cycles of 94°C for 30 s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. A 421 bp fragment was obtained and cloned into the expression vector pEF-BOS, as shown in Figure 19b. The open reading frame and FLAG fusion were confirmed by sequencing.

HEK 293 cells were transfected with both mouse and rat expression constructs using Lipofectamine (Life Technologies, Inc). Supernatant was harvested after 72 hours. The recombinant protein was purified by incubation with the anti-FLAG M2 affinity gel (Kodak), and eluted with the FLAG peptide (Kodak) as outlined in the manufacturer's protocol. The purified protein was used to

study its effects on osteoclast formation in murine cocultures.

An experiment was also carried out to determine the action of rOCIL protein in osteoclast formation. Primary mouse calvarial osteoblasts were cocultured with spleen or bone marrow cells obtained from 6 week-old mice and stimulated with 1,25-dihydroxyvitamin D_3 and PGE_2 in the presence of rOCIL or mOCIL protein (15 ng/ml) for 10 days. A negative control was carried out with carrier buffer alone. As shown in Figure 20, both rOCIL protein (Figure 20a) and mOCIL protein (Figure 20b) significantly reduced the number of osteoclasts formed when compared to the presence of carrier buffer alone.

Example 12: Effect of mOCIL Protein on Osteoclast Formation

To determine the action of mOCIL protein on osteoclast formation, mouse spleen cells were obtained from 6-week old mice and cultured in medium containing 10% FCS, 25ng/ml hM-CSF and 50ng/ml murine soluble RANKL in the absence or presence of mOCIL protein at a concentration of 12.5 ng/ml. mOCIL protein treatment resulted in a 60% inhibition of sRANKL and hM-CSF stimulated osteoclast formation, as illustrated in Figure 21.

Example 13: Production of Recombinant OCIL protein in an E.coli expression system

In order to increase the expression level for mOCIL protein, an *E.coli* expression system was used. A DNA fragment encoding the extracellular domain (residues 76-207) of mOCIL was obtained by PCR and cloned into the *Eco*RI and *Hin*dIII site of pMAL-c2 (New England Biolabs Inc.), creating a gene fusion with the MBP (maltose binding protein)-encoding *mal*E gene. PCR was performed using a plasmid which contained mOCIL17 cDNA sequence (SEQ ID NO: 17) as a template. The reaction used a sense primer representing nucleotides 285-303 of mOCIL (SEQ ID NO: 36)

encoding amino acids 76-81, TYAACP, in SEQ ID NO: 39 with an *Eco*RI site, designated primer OCILm65 (SEQ ID NO: 50),

OCILm65 5'-TCAGAATTCACCTATGCTGCTTGCCCGC-3'

and an antisense primer representing nucleotides 711-690 of mOCIL after the stop codon in SEQ ID NO: 36 with a *Hin*dIII site, and designated primer OCILm66 (SEQ ID NO: 51):

OCILm66 5'-GGTTAAGCTTCAGGCTAAAAAGCGTCTCTTGG-3'.

PCR was run at 94°C for 5 min, then 30 cycles of 94°C for 30 s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. The PCR product was then digested with *Eco*RI and *Hin*dIII, and cloned into pMAL-c2, as shown in Figure 22. The open reading frame and MBP fusion were confirmed by sequencing.

Example 14: Recombinant mOCIL-related protein constructs

To determine whether mOCIL-related proteins also have an inhibitory effect on osteoclast formation, the sequences encoding the extracellular domains of mOCILrP1 and mOCILrP2 were also inserted into the *Eco*RI and *Hin*dIII sites of pMAL-c2 (New England Biolabs Inc.), as described above.

PCR was performed using a plasmid which contained mOCILrP1 cDNA sequence (SEQ ID NO: 12) as a template. A sense primer representing nucleotides 283-302 of mOCILrP1 (SEQ ID NO: 12) encoding amino acids 88-93, TYAACP, in SEQ ID NO: 40 with an *Eco*RI site, designated primer OCILm88 (SEQ ID NO: 52),

OCILm88 5'-TCAGAATTCACCTATGCTGCTTGCCCGAA-3'

and an antisense primer representing nucleotides 742-720 of mOCILrP1 (SEQ ID NO: 12) and 739-717 of mOCILrP2 (SEQ ID NO: 15), with a *Hin*dIII site, and designated primer OCILm87

(SEQ ID NO: 53)

OCILm87 5'-GGTTAAGCTTGGGACCATAGGGGAAAAAGTAG-3'

were constructed. PCR was run at 94°C for 5 min, then 30 cycles of 94°C for 30 s, 60°C for 30s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. The PCR product was then digested with *Eco*RI and *Hin*dIII and cloned into pMAL-c2 (Figure 23). The open reading frame and MBP fusion were confirmed by sequencing.

For the mOCILrP2 construct, PCR was performed using a plasmid which contained mOCILrP2 cDNA sequence (SEQ ID NO: 15) as a template. A sense primer, representing nucleotides 283-302 of mOCILrP2 (SEQ ID NO: 15) encoding amino acids 88-93, TYAACS of SEQ ID NO: 41 with an *Eco*RI site, designated primer OCILm89 (SEQ ID NO: 54),

OCILm89 5'-TCAGAATTCACCTATGCTGCTTGCTCAAA-3'

and antisense primer OCILm87 (SEQ ID NO: 53) were used for PCR. PCR and cloning procedures were carried out under the same conditions as above, as shown in Figure 24.

Example 15: Recombinant hOCIL protein construct

A recombinant hOCIL protein construct was also made using the same system. A sense primer, representing nucleotides 694-711 of hOCIL clone 1 (SEQ ID NO: 20) with an *Eco*RI site, designated primer hpMAL-1 (SEQ ID NO: 55),

hpMAL-1 5'-GCGGAATTCCTTCAAGCTGCATGCCC-3'

and an antisense primer representing nucleotides 1034-1055 of hOCIL clone 1 (SEQ ID NO: 20) with a *Bam*HI site, and designated primer hpMAL-2 (SEQ ID NO: 56)

hpMAL-2 5'-CCTGGGATCCGCTTTGCTGTAACATCTAGAC-3'

were used to run PCR under the same conditions as above. The PCR product was then digested with *Eco*RI and *Bam*HI, and cloned into pMAL-c2. The open reading frame and MBP fusion was confirmed by sequencing, as shown in Figure 25.

Example 16: Expression and purification of mOCILrP and hOCIL

Competent strain BL21 *E.coli* cells were transformed with the constructs produced in the preceding two examples, and the corresponding fusion protein was induced with IPTG (isopropyl-1-thio-β-D-galactopyranoside) according to the manufacturer's instructions. The MBP-OCIL fusion protein was isolated from the soluble bacterial fraction using affinity chromatography as outlined in the manufacturer's instructions. The eluant fractions were subjected to SDS-PAGE, and transferred to membranes [PVDF (polyvinylidene difluoride) Western blotting membranes (Roche Molecular Biochemicals)]. Western blot analyses were performed with a rabbit anti-MBP serum (New England Biolabs Inc.) and a BM chemiluminescence blotting substrate (POD) detection system (Roche Molecular Biochemicals). Fractions containing the MBP-OCIL fusion protein were pooled and concentrated using an Amicon ultrafiltration YM10 membrane (Millipore, Bedford, MA). The protein concentration was ascertained in a BCA protein assay (Pierce). The estimated yield of MBP-mOCIL ranged between 0.1-0.4 mg/L.

Example 17: Inhibition of osteoclast formation

Experiments were carried out to determine the action of MBP fusion OCIL proteins on osteoclast formation.

Mouse culture system

Mouse spleen cells obtained from 6 week old adult mice were cultured in medium containing 10% FCS and 25ng/ml M-CSF and 50ng/ml sRANKL in the absence or presence of MBP or MBP-mOCIL fusion protein at various concentrations. After 7 days, cells were fixed and subjected to TRAP

staining. As shown in Figure 26a, MBP-mOCIL fusion protein significantly reduced the number of osteoclasts formed when compared to MBP protein alone, and this effect was shown in a dose-dependent manner.

It has been reported that IL-18 inhibits osteoclast formation mediated by T cells (Horwood *et al.*, 1998). To further investigate whether the mOCIL inhibition of osteoclast formation is a T cell-dependent effect, T cell-depleted mouse spleen cell cultures were carried out as reported by Horwood *et al.*, (1998). T cells were depleted with CD3 antibody from spleen cells and the remaining cells were cultured in medium containing 10% FCS, 25ng/ml M-CSF and 50ng/ml sRANKL in the absence or presence of MBP or MBP-mOCIL fusion protein at a concentration of 500ng/ml. The results, summarised in Figure 26b, showed that mOCIL inhibited osteoclast formation, implying that its actions were T-cell independent.

The effect of MBP-mOCILrP1 and MBP-mOCILrP2 fusion protein on osteoclast formation was also examined in the T-cell depleted mouse spleen cell culture system. The results are shown in Figure 27. Both MBP-mOCILrP1 (Figure 27a) and MBP-mOCILrP2 fusion proteins (Figure 27b), like mOCIL, inhibited osteoclast formation in a T cell independent fashion.

Human monocyte culture

Monocyte cultures were prepared as described by Quinn et al., (1998). Monocytes were isolated from the peripheral blood of normal healthy subjects. Human PBMCs were prepared from diluted blood (1:1 in Hanks Balanced Salt Solution (HBSS; Life Technologies, Grand Island, NY) which was layered over Ficoll-Paque® solution (Pharmacia Biotech, Uppsala, Sweden), centrifuged (693g), then washed and resuspended in MEM medium containing 10% FCS. Monocyte cultures were prepared by adding 10^6 PBMCs to 6mm diameter culture wells containing bovine cortical bone slices in MEM medium containing 10% FCS; after 1 hour, coverslips and bone slices were removed, vigorously rinsed to remove non-

adherent cells, and placed in 10mm diameter culture wells. Monocyte cultures were maintained in these culture wells in 0.4ml MEM medium containing 10% FCS, recombinant human M-CSF (25ng/ml) and recombinant human sRANKL (30 ng/ml) in the absence or presence of MBP (500 ng/ml) or MBP-hOCIL (500ng/ml) fusion protein for 21 days. Medium and added factors were entirely replaced every 3 days. After 21 days, bone slices were removed for TRAP staining and bone resorption pit analysis. The multinucleate osteoclasts were counted, and the results are shown in Table 5. Human OCIL inhibited osteoclast formation from human monocytic cells.

Table 5

Effects of MBP-hOCIL fusion protein on osteoclast formation in human monocyte cultures

Well	Control(RANKL+M-CSF)	MBP (500 ng/ml)	MBP-hOCIL (500 ng/ml)
1	205	40	6
2	146	180	16
3	37	17	34
4	66	42	9
5	66	22	73
6	71	63	14
7	38	54	20
8	25	29	34
Mean ± SEM	81.7 ± 22.0	55.9 ± 18.6	26.12 ± 7.62

DISCUSSION

We conclude that in osteoblasts OCIL and OCIL related proteins are expressed on the cell surface as type II membrane peptides. Contact with haematopoietic precursor cells prevents further differentiation into mononucleate osteoclast precursors, and ultimately into functional multinucleate osteoclasts.

Without wishing to limit the scope of the invention by any proposed mechanism, we consider that upregulation of OCIL mRNA expression by the same osteotropic factors that increase expression of RANKL is consistent with the hypothesis that regulation of bone resorption by osteoclasts is tightly regulated. According to this hypothesis, stimulation of multinucleate osteoclast formation through RANKL would simultaneously prevent the generation of new osteoclasts through the action of OCIL. If this system is operative under normal physiological conditions, then bone resorption becomes a self-limiting process.

Notwithstanding the above, mOCILrP1 and mOCILrP2 are not regulated by osteotropic agents that regulate mOCIL, but like mOCIL, both mOCILrP1 and mOCILrP2 have the capacity to inhibit osteoclast formation. Thus each of the three polypeptides, mOCIL, mOCILrP1 and mOCILrP2 is equally useful for therapy to limit osteoclast formation or to promote osteoclast formation through blockade of their actions. Given the degree of homology between these molecules, each may substitute for one another. However, each can be distinguished by several criteria. These include:

(a) Nucleotide sequence: mOCIL, mOCILrP1 and mOCILrP2 appear to be derived from a common ancestral gene; however, there are nucleotide differences which permit identification of the three molecules using specific oligonucleotide primers in RT-PCR.

(b) Gene structure: The promoter of mOCIL is a TATA promoter, while the promoter for mOCILrP1 is a GC-rich

region containing an SP 1 binding site.

(c) The expression of mOCIL is regulated by PTH, while the expression of mOCILrP1 and mOCILrP2 is not.

(d) The polypeptide products of mOCIL, mOCILrP1 and mOCILrP2 can be distinguished using antibodies directed against peptides fragments of mOCIL and mOCILrP1/rP2 based on the intracellular domains of the respective proteins.

In vivo, OCIL and OCILrP have the potential to be used as therapeutic agents in the treatment of conditions which are characterised by excessive bone resorption, such as osteoporosis, primary hyperparathyroidism, Paget's disease, rheumatoid arthritis, renal osteodystrophy, and humoral hypercalcaemia of malignancy, as well as metastatic bone disease. Modulation of the expression or function of the factor may also be useful in the treatment of disorders involving extra-skeletal calcification.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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